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(54) Leucine aminopeptidase gene, recombinant dna, and process for producing leucine aminopeptidase

(57) The present invention relates to an *Aspergillus sojae* leucine aminopeptidase gene or variants thereof, a recombinant DNA including the gene or the variants, and a process for producing leucine aminopeptidase using the recombinant DNA

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Solution B: an aqueous solution of 0.4%(w/v) trinitrobenzenesulfonic acid (TNBS)
Solution C: an aqueous solution of 5%(w/v) $Na_2B_4O_7 \cdot 10H_2O$
Solution D: an aqueous solution of 100 mM $CuSO_4$
Solution E: a mix of 250 μl of Solution B. 975 μl of Solution C and 25 μl of Solution D per specimen (prepared upon use)

[0017] Then, two microcentrifuge tubes per specimen are each filled with 25 μl of the enzyme solution. One of the two tubes is heated at 100°C for 5 min. to inactivate the enzyme for use as a blind test sample. To each microcentrifuge tube, 475 μl of Solution A (at 30°C) is added and stirred prior to carrying out the enzyme reaction at 30°C for 10-60 min. The reaction is terminated by heating at 100°C for 5 min. To the reaction mixture, 625 μl of Solution E (at 37°C) is added and stirred. After keeping the mixture at 37°C for 25 min., an absorbance is measured at 420 nm. Providing that 1 unit equals to an activity for freeing 1 μmol of Leu per min. from Leu-Gly-Gly, the leucine aminopeptidase activity can be calculated by the following equation:

$$\text{Activity (unit/ml)} = (\Delta OD \times 0.125 \times 1{,}000)/(25 \times T)$$

wherein ΔOD is obtained by subtracting an OD value of the blind test sample from an OD value of the test sample; and T is a reaction time (in min.).

Method 2: Assay using L-leucine-p-nitroanilide (Leu-pNA) as a substrate

[0018] First, the following solutions are prepared.

Solution A: 100 ml of 1 mM Leu-pNA solution which is obtained by dissolving 0.1 mmol of Leu-pNA in 5 ml of ethanol and then adding 10 ml of 500 mM Tris buffer (pH 8.5) and 85 ml of distilled water
Solution B: 0.1 N HCl

[0019] Then, a microcentrifuge tube containing 30 μl of the enzyme solution and a blank microcentrifuge tube for blind test are each filled with 300 μl of Solution A and stirred prior to carrying out the enzyme reaction at 30°C for 10-60 min. 900 μl of Solution B is added and stirred in order to terminate the enzyme reaction. To the microcentrifuge tube for blind test, 30 μl of the enzyme solution is added and stirred. An absorbance is determined at 400 nm. Providing that 1 unit equals to an activity for freeing 1 μmol Leu per min. from Leu-pNA, the leucine aminopeptidase activity can be calculated by the following equation:

$$\text{Activity (unit/ml)} = (\Delta OD \times 0.69 \times 1{,}000)/(30 \times T)$$

wherein ΔOD is determined by subtracting an OD value of the blind test sample from an OD value of the test sample; and T is a reaction time (in min.).

Method 3: Simple assay for detecting abilities of various strains to produce leucine aminopeptidase

[0020] Solutions A and B in Method 2 are used as reagents.

[0021] Ten μl of sterilized distilled water or an aqueous solution of 0.01%(w/v) Tween-20, containing about 1,000 spores from a filamentous fungous, e.g., *Koji mold Aspergillus sojae*, is applied to a thick paper disk (8 mm φ ; Toyo Roshi Co., Ltd.) placed on a soybean powder agar medium [3%(w/v) defatted soybean powder obtained by swelling under heat and pressure conditions, 1%(w/v) KH_2PO_4, 1.5% agar powder, pH6.0]. A blind test is concurrently conducted in the same manner, except that there are contained no spores. They are cultured at 30°C until sporulation begins (for about 48 hours in the case of *Koji*). Each paper disk with the cell is transferred to a test tube (10 mm diameter or larger), to which 600 μl of Solution A is added at 30°C. The mixture is then thoroughly stirred prior to carrying out the enzyme reaction at 30°C for 7-30 min. Then, to the reaction mixture, 600 ml of Solution B is added and vigorously stirred, after which an absorbance is determined at 400 nm. The arbitrary unit of the leucine aminopeptidase activity is determined by subtracting an absorbance of the blind test sample from that of the test sample. However, since this method gives widely varied results, it is desirable to conduct three or more simultaneous tests per strain. Moreover, strains that are to be compared to each other should be subjected to simultaneous assay. When an organism other than filamentous fungi is used, it may be assayed in a similar way by suitably modifying the number of cells, a medium composition and a culture time.

yeasts insect cells plant cells or animal cells preferably filamentous fungi for example *Aspergillus sojae* ATCC42251 according to methods described in J. Sambrook et al. Molecular Cloning Second Edition Cold Spring Harbor Laboratory Press, 1989 E. Shiela et al., Molecular & General Genetics, 218, 99-104 (1989); etc. In the transformation method in which a filamentous fungous is used as a host cell the cultured cell is treated with a cell-wall degrading enzyme such as Novozym 234 (Novo Nordisk A/S) to obtain a protoplast in which the cell-wall has been removed Then DNA containing a marker gene such as niaD, and DNA containing a gene of interest, are concurrently introduced into the protoplast in the coexistence of calcium chloride and polyethyrene glycol 4000. Thereafter, the so-treated protoplast is diluted in a selection medium suitable for the used marker gene and then is kept warm, whereby a transformant in which both the marker gene and the gene of interest have been integrated in the chromosome of the host cell can be regenerated. The leucine aminopeptidase gene may be a recombinant DNA inserted into a vector, or a non-recombinant DNA such as a DNA fragment amplified from a chromosomal DNA by PCR. In the latter case, transformation can be performed without troubles because even the non-recombinant DNA can be integrated in the chromosome of a host cell. The obtained transformants may be screened for an enhanced ability to produce leucine aminopeptidase as measured by, for example, Method 3 described above, to obtain a transformant of interest.

[0032] The thus-obtained transformant can be cultured according to the method described in JP-A-48-35094 to produce and purify leucine aminopeptidase, resulting in efficient production of leucine aminopeptidase.

EXAMPLES

[0033] Hereinafter, the present invention will be illustrated in more details by the following non-limited examples

Example 1: Cloning of leucine aminopeptidase gene

(1) Purification of enzyme

[0034] *Koji mold Aspergillus sojae* 1-190 (FERM BP-6349) was cultured to produce and purify leucine aminopeptidase as described above (3 mg yield).

(2) Determination of partial amino acid sequence

[0035] 30 ng of the purified enzyme was used as a sample and the N-terminal amino acid sequence thereof was determined to be Gly Arg Ala Leu Val Ser Pro Asp Glu Phe Pro (SEQ ID NO:3) by using ABI470A Protein Sequencer (Perkin-Elmer). However, since this enzyme allows an amino acid to be released from the N-terminus of a peptide, it was impossible to avoid contaminants partially lacking the N-terminus by autolysis, resulting in that no sequence other than the above-determined sequence was obtained.

[0036] Instead, the enzyme was fragmented with lysylendpeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to determine its internal amino acid sequence There, however, still remained the problem of amino acids release from the N-termini of peptide fragments due to the remaining enzyme activity.

[0037] Through further examinations, the present inventors have found that the enzyme was fragmented under such conditions that inactivate the enzyme but allows lysylendpeptidase to function. First, 1 mg of the purified enzyme was inactivated at 37°C for 1.5 hours in 0.5 ml of 50 mM Tris-buffer (pH 9.0) containing 1%(w/v) SDS and 5 mM EDTA. After the inactivation, 15 µl of 0.1 mg/ml lysylendpeptidase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added for the reaction at 37°C for 14 hours. Five µl of the lysylendpeptidase was further added to continue the reaction at 37°C for 2 hours. The thus-obtained solution containing peptide fragments was added to 55 µl of 1 M potassium phosphate buffer (pH 7.5), left on ice for 30 min., and centrifuged at 15,000 x g for 20 min. to remove SDS. From this peptide fragment solution, solid substance was removed using Column Guard HV 13mm (Millipore). To 100 µl of the prepared peptide fragment solution, trifluoroacetic acid (hereinafter referred to as "TFA") was added to a final concentration of 0.1% in order to perform a reversed-phase chromatography with a gradient from 0 to 47.5% acetonitrile in 0.1% TFA-containing ultrapure water using POROS R2/H Prepacked Column (Boehringer Mannheim). Each peptide peak was fractionated monitoring an absorbance at 220 nm as the indication. The peptide solution obtained at each peak was evaporated to dry with a vacuum centrifugal concentration system, and re-dissolved in 20 µl of 20 mM phosphate buffer (pH 8.0). Amino acid sequences of these peptides were determined using ABI470A Protein Sequencer (Perkin-Elmer). As a result, peptide at Peak 5 was Xaa Xaa Xaa Xaa Xaa Xaa Xaa Asp Tyr Pro Ser Val Glu Gly Lys (where Xaa is an amino acid that was unable to be identified due to contamination of other peptide) (SEQ ID NO:4), peptide at Peak 6 was Gln Pro Gln Val His Leu Trp ... (SEQ ID NO:5); and peptide at Peak 11 was Asn Ala Val Arg Phe Leu Phe Trp Thr Ala Glu Glu Phe Gly Leu Leu Gly Ser Asn Tyr Tyr Val Ser His Leu ... (SEQ ID NO:6).

of (ii) 95°C, 30 sec., (iii) 50°C, 30 sec. and (iv) 72°C, 40 sec.; and thereafter (v) 72°C, 2 min. The reaction mixture was electrophoresed on 1% agarose gel to recover an amplified fragment of about 500 bp using QIAquick Gel Extraction kit (Qiagen). The amplified fragment was eluted with 50 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

[0046] Digoxigenin (DIG) -labeled DNA probe was prepared through PCR as follows: using FOW-LAP-A and REV-LAP-A as primers and a 10-fold dilution of the above amplified fragment solution as a template, 37.75 μl of sterilized distilled water, 5 μl of 10 x buffer, 1 μl each of the two primer DNA solutions (100 μmol/μl), 1 μl of template DNA solution, 4 μl of 10 x PCR DIG mix (Boehringer Mannheim) and 0.25 μl of ExTaq DNA polymerase were put and mixed in a 0.2-ml PCR tube. 20 μl of mineral oil was added dropwise to the mixture before setting the tube in RoboCycler Gradient 96. The genomic DNA was previously denatured at 96°C for 3 min., then rapidly cooled on ice. The reaction was conducted while programming the RoboCycler Gradient 96 as follows: (i) 95°C, 30 sec., 45 cycles of (ii) 95°C, 30 sec., (iii) 62°C, 30 sec. and (iv) 72°C, 40 sec.; and thereafter (v) 72°C, 2 min. The amplified fragment was collected from the reaction mixture by ethanol precipitation, and dissolved in 50 μl of TE buffer. This was named as DIG-labeled probe dLAP-A.

(6) Screening of leucine aminopeptidase gene by plaque hybridization

[0047] The λ phage library of the *Kcji* genomic DNA prepared in (4) was screened for the leucine aminopeptidase gene by using the probe of (5).

[0048] According to the instructions appended to the kit for preparing the library, approximately 5 x 10^3 of plaques per plate were formed on 5 agar medium plates. From each plate, the DNA was transferred to HyBond-N+ Nylon Transfer Membrane (Amersham) according to the instructions appended to the membrane. In this case, for elimination of non-specific signals, the DNA was transferred to two membranes per plate individually.

[0049] Those membranes were subsequently subjected to hybridization and detection by using the digoxygenin-labeled DNA probe prepared in (5) and DIG system (Boehringer Mannheim) according to "Users Guide for hybridization using DIG system", pages 37-40, Boehringer Mannheim (1996). Specifically, a standard pre-hybridization buffer (5 x SSC, 1 % (w/v) nucleic acid hybridization blocking reagent (Boehringer Mannheim), 0.1% (w/v) N-lauroyl sarocosine, and 0.02% (w/v) SDS) was applied to each of the above ten membranes on which the DNAs of the phage library have been adsorbed, and the membranes were left at 57°C for 1 hour while shaking gently.

[0050] Thereafter, the dLAP-A probe prepared in (5), which has been heated at 100°C for 10 min and rapidly cooled in ice water, was applied to each membrane which was then left at 57°C for 16 hours while shaking gently. Each membrane was transferred to a new container and washed twice with 2 x washing solution (2 x SSC, 0.1% (w/v) SDS) at room temperature for 5 min., and subsequently washed twice with 0.1 x washing solution (2 x SSC, 0.1% (w/v) SDS) at 57°C for 15 min. Each membrane was then equilibrated with Buffer 1 (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 1 min., and transferred to a new container, to which Buffer 2 (Buffer 1, 1%(w/v) nucleic acid hybridization blocking reagent) was added, followed by gently shaking at room temperature for 30 min. After discarding Buffer 2, an alkaline-phosphatase-labeled anti-digoxygenin antibody diluted 1:10,000 with Buffer 2 was added and gently shaken at room temperature for 30 min. After discarding the antibody solution, the membranes were individually washed twice with Buffer 1 containing 0.3% Tween 20 in a clean container. Then, they were equilibrated with Buffer 3 (0.1M Tris pH9.5, 0.1 M NaCl, 50 mM $MgCl_2$) for 2 min. Each of the membranes was placed in a hybridization bag cut into a suitable size, to which 0.4 ml of 100-fold diluted CDP-Star solution (Boehringer Mannheim) was added dropwise, thereby being uniformly distributed over the membrane. After removal of the excessive solution, the bags were sealed with a sealer. These membranes were kept at 37°C for 10 min. and then closely attached to Fuji direct photography films RX-U (Fuji Photo Film Co., Ltd.) for 1-10 min., which films were then developed. Five signals (named Signals A to E) were observed at the same location on the two membranes to which the plaques on the same agar medium plate have been transferred. A to E might be positive clones. Soft agar portions (5 mm diameter) were removed from the positions corresponding to Signals A to E and put in respective microcentrifuge tubes. After 500 μl of SM buffer (10 mM NaCl, 0.2% (w/v) $MgSO_4 \cdot 7H_2O$, 50 mM Tris pH7.5, 0.01% gelatin) and 20 μl of chloroform were added, the tubes were left at 4°C overnight. While being controlled such that about 100 phages per tube were produced, plaques were formed on these agar media. They were transferred to respective membranes as described above in order to perform hybridization with dLAP-A probe. For the phage solution obtained from Signal A, a number of strong signals were observed. While no signal was observed for Signal B, a few signals were observed for Signals C, D and E. Plaques were collected from Signals A, C, D and E as described above to perform plaque hybridization again. Since all plaques of Signals A and D turned out to be positive, single plaques were recovered individually from Signals A and D.

[0051] The phage clones of Signals A and D were subjected to *in vitro* excision according to the instructions appended to the kit to obtain their plasmids, which were named pLZA and pLZD, respectively.

Example 3 Production of leucine aminopeptidase using transformant

[0063] 2.78 g of wheat bran and 2.22 ml of distilled water were mixed, put in a 150-ml Erlenmeyer flask, autoclaved at 121°C for 50 min., and left to cool to room temperature. 10^6 spores each of the host strain and the three transformant strains were inoculated into respective flasks and left at 30°C for 24 hours. The flasks were vigorously shaken to break the contents into small particles, which were left at 30°C for another 48 hours for growth of the cells. 25 ml of distilled water was added to the flasks which were then vigorously shaken and left at room temperature for 3 hours. Enzymes were extracted from the mixtures, thereby obtaining enzyme solutions.

[0064] In order to determine aminopeptidase activities of the enzyme solutions, free amino acids and low-molecular peptides were removed from the enzyme solutions. Specifically, 2 ml of each enzyme solution was put in Centricon-10 (Amicon) and centrifuged at 3,000 x g. When the amount of the liquid became 0.5 ml, 1.5 ml of 25 mM HEPES buffer (pH 7.0) was added thereto and the mixture was centrifuged three times at 3,000 x g. The volume of the collected enzyme solution was adjusted to 2 ml with 25 mM HEPES buffer (pH 7.0).

[0065] The leucine aminopeptidase activities of the thus-obtained enzyme samples were determined using Leu-Gly-Gly as a substrate (Table 1). Defining the activity of the host strain as 1, the activities of TFLW14, TFLW5 and TFLW22 were about 1.0, about 2.1 and about 4.9, respectively. Leucine aminopeptidase was efficiently produced by growing the transformant TFLW22 transformed with the vector containing the leucine aminopeptidase gene, as compared to the host strain.

Table 1

	Leucine aminopeptidase activity (U/g *Koji*)	Ratio of activity to that of host strain
ATCC42251 niaD-	1.14	1.0
TFLW14	1.18	1.0
TFLW5	2.41	2.1
TFLW22	5.56	4.9

[0066] According to the present invention, leucine aminopeptidase can efficiently be obtained by culturing, for example, a microorganism containing the recombinant DNA into which the leucine aminopeptidase gene of the invention has been incorporated. Since the gene of the invention may be used as a sample for protein engineering, the present invention is industrially useful.

[0067] Various other modifications will be apparent to and can be readily made by those skilled in the art without departing from the scope and spirit of this invention. Accordingly, it is not intended that the scope of the claims appended hereto be limited to the description as set forth herein, but rather that the claims be broadly construed.

[0068] All publications including patent applications cited herein are incorporated herein by reference in their entirety.

[0069] The following are information on SEQ ID NOS: 1 and 2 described herein:

165 170 175

Ile Val Gly Ile Ser Leu Glu Asp Gly Gln Lys Leu Ile Lys Leu Ala

180 185 190

Glu Ala Gly Ser Val Ser Val Asp Leu Trp Val Asp Ser Lys Gln Glu

195 200 205

Asn Arg Thr Thr Tyr Asn Val Ile Ala Gln Thr Lys Gly Gly Asp Pro

210 215 220

Asn Asn Val Val Ala Leu Gly Gly His Thr Asp Ser Val Glu Ala Gly

225 230 235 240

Pro Gly Ile Asn Asp Asp Gly Ser Gly Ile Ile Ser Asn Leu Val Val

245 250 255

Ala Lys Ala Leu Thr Gln Tyr Ser Val Lys Asn Ala Val Arg Phe Leu

260 265 270

Phe Trp Thr Ala Glu Glu Phe Gly Leu Leu Gly Ser Asn Tyr Tyr Val

275 280 285

Ser His Leu Asn Ala Thr Glu Leu Asn Lys Ile Arg Leu Tyr Leu Asn

290 295 300

Phe Asp Met Ile Ala Ser Pro Asn Tyr Ala Leu Met Ile Tyr Asp Gly

305 310 315 320

Asp Gly Ser Ala Phe Asn Gln Ser Gly Pro Ala Gly Ser Ala Gln Ile

325 330 335

Glu Lys Leu Phe Glu Asp Tyr Tyr Asp Ser Ile Asp Leu Pro His Ile

340 345 350

Pro Thr Gln Phe Asp Gly Arg Ser Asp Tyr Glu Ala Phe Ile Leu Asn

355 360 365

Gly Ile Pro Ala Gly Gly Leu Phe Thr Gly Ala Glu Gly Ile Met Ser

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35 40 45

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50 55 60

Tyr Lys Gln Pro Gln Val His Leu Trp Ser Asn Ala Asp Gln Thr Leu

65 70 75 80

Lys Val Gly Asp Glu Glu Ile Glu Ala Lys Thr Met Thr Tyr Ser Pro

85 90 95

Ser Val Glu Val Thr Ala Asp Val Ala Val Val Lys Asn Leu Gly Cys

100 105 110

Ser Glu Ala Asp Tyr Pro Ser Asp Val Glu Gly Lys Val Ala Leu Ile

115 120 125

Lys Arg Gly Glu Cys Ala Phe Gly Asp Lys Ser Val Leu Ala Ala Lys

130 135 140

Ala Lys Ala Ala Ala Ser Ile Val Tyr Asn Asn Val Ala Gly Ser Met

145 150 155 160

Ala Gly Thr Leu Gly Ala Ala Gln Ser Asp Lys Gly Pro Tyr Ser Ala

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Glu Ala Gly Ser Val Ser Val Asp Leu Trp Val Asp Ser Lys Gln Glu

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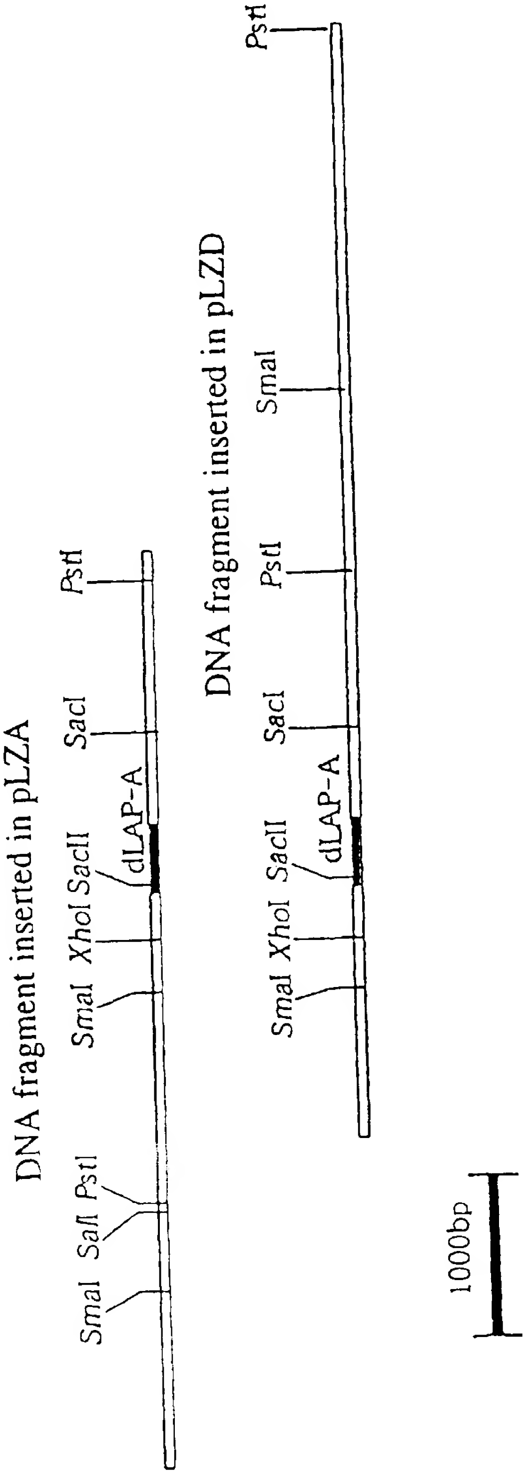
FIG.1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SalI
PstI
SmaI
XhoI
SacII
dLAP-A
SacI
PstI
DNA fragment inserted in pLZD
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XhoI
SacII
dLAP-A
SacI
PstI
SmaI
PstI
1000bp